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High-efficiency filter fluorometer for capillary electrophoresis and its application to fluorescein thiocarbamyl amino acids

Edgar Arriaga, Da Yong Chen, Xiao Li Cheng and Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2 (Canada)

ABSTRACT

Fluorescence detection has produced excellent detection limits in capillary electrophoresis. Laser excitation produces the highest sensitivity detection. The perceived difficulties associated with the use of the laser has discouraged applications of fluorescence to capillary electrophoresis. In particular, difficulties in wavelength selection limit the choice of chemistry available in capillary electrophoresis. We report the design of a filter fluorometer based on a compact 75 W xenon arc lamp for capillary electrophoresis; the instrument produces detection limits (3σ) of 20 zeptomol (1 zeptomol = 10^{-21} mol) for fluorescein and 200 zeptomol for fluorescein labeled amino acids.

INTRODUCTION

Laser-based fluorescence detection has produced spectacular results for capillary electrophoresis [1–25], with state-of-the-art detection limits in the yoctomol ($= 10^{-24}$ mol) range [22,24]. While lasers produce spectacular detection performance in fluorescence instrumentation, they suffer from a number of perceived limitations, including high cost and limited lifetime. In reality, the limited choice of excitation wavelengths may be the most important limitation of lasers [26].

The performance of fluorometers built with incoherent excitation sources tends to be many orders of magnitude poorer than the laser-based systems [27–32]. The best results for non-laser-based fluorescence detection came from Jorgenson's group, who reported detection limits of 83 amol (1 attomol = 10^{-18} mol) for *o*-phthalaldehyde–glycine excited with a mercury–xenon arc lamp in a post-column detector [29]. Wallingford and Ewing [30] used a 200-W mercury lamp for fluorescence excitation, but they did not report detection limits. Commercial instruments have used a pulsed xenon flash lamp, focused directly onto the capillary, for fluorescence detection [31]. Workers from Applied Biosystems compared xenon, deuterium and tungsten lamps for fluorescence excitation [32]. That system employed a monochromator to select excitation wavelength, an aperture to control the size of the illuminated region in the capillary, fiber optics to collect fluorescence, and an emission filter placed before a photomultiplier tube detector. A xenon lamp produced the best detection limits, 500 attomoles of 9-fluorenylmethyl chloroformate–glycine.

At first glance, use of an incoherent arc lamp fluorometer appears difficult in capillary electrophoresis. The very small excitation volumes required in capillary separations (less than 1 nl) and the stringent requirements on light scatter are challenging constraints. However, recent work in this laboratory has provided evidence that lamp-based excitation should be possible with high sensitivity. In particular, we have found that low-power coherent excitation

* Corresponding author.

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sources can produce yoctomole detection limits in fluorescence instrumentation; an instrument based on a 0.75 mW helium neon laser (λ = 543.5 nm) produces detection limits of 500 tetramethylrhodamine molecules [24]. Theory suggests that a xenon arc lamp in a filter fluorometer will produce 0.75 mW of optical power at the sample.

In this paper, we report a filter fluorometer based on a xenon arc lamp. This instrument produces excitation from the near ultraviolet at 250 nm through the infrared; an appropriate bandpass interference filter isolates the desired wavelength. The system is designed to excite fluorescence efficiently. A 75-W xenon arc lamp, with an integral parabolic reflector, efficiently couples light to the experiment. An aspheric lens efficiently illuminates a limiting aperture; this aperture blocks stray light from the lamp and defines the excitation volume. A high-quality microscope objective images the aperture onto a sheath flow cuvette. The sheath flow cuvette has excellent optical properties, resulting in background scatter that is lower than would be produced with on-column detection. A high-quality microscope objective collects fluorescence from the sample and images it onto an adjustable aperture. This aperture blocks stray light in the instrument, decreasing the background signal.

We chose to use an interference filter to isolate fluorescence rather than a monochromator. In our experience, it is much easier to achieve much higher transmission through a filter compared with the monochromator. We use an aperture to define the illumination volume rather than imaging the entire source onto the sample; light scatter is reduced with this aperture. We use a sheath flow cuvette rather than direct illumination of the capillary; light scatter is minimized. We use a high-efficiency microscope objective to collect fluorescence rather than fiber optics. The objective generates an excellent image of the excited sample; stray light is easily blocked with an aperture placed in the image plane of the objective.

THEORY

Consider a 75-W xenon arc lamp that acts as black-body radiator at 5600 K. Plank's black-body radiation equation may be written as

$$E_T(\lambda)\,d\lambda = \frac{2C^2h}{\lambda^5}\frac{d\lambda}{e^{hc/\lambda kT}-1} = \frac{1.19\cdot 10^{-16}\ \mathrm{W\,m^{-2}\,steradian^{-1}}}{\lambda^5} \cdot \frac{d\lambda}{e^{1.44\cdot 10^{-2}(Km)/\lambda T}-1} \quad (1)$$

where E_T is the spectral radiance of a surface in units of W m^{-2} steradian^{-1} over the spectral region $d\lambda$, λ is wavelength in m, C is the speed of light, h is Planck's constant, k is Boltzmann's constant and T is absolute temperature [33]. Table I lists the spectral radiance, integrated across a 10-nm spectral band-width for 5600 K black body.

Light from the lamp must be imaged onto the sample stream with an optical system. If the arc is imaged directly into the cuvette, a large amount of stray light will be generated. To reduce this stray light, the arc is first imaged onto an aperture; this limiting aperture is then imaged onto the cuvette.

From the second law of thermodynamics, the radiance at the image of the lamp will be less than or equal to the radiance of the lamp itself. In a filter fluorometer for capillary electrophoresis, the lamp is assumed to illuminate a 50-μm radius region in a fluorescence detection chamber. The optical power, P, that is available to illuminate the detection chamber is then given by

TABLE I

SPECTRAL RADIANCE OF A 5600-K BLACK BODY IN A 10-nm SPECTRAL BAND

λ (nm)	250	300	400	500	600	700
E (W m^2 steradian^{-1})	42 200	93 900	190 000	225 000	215 000	185 000

TABLE II

POWER DELIVERED TO SAMPLE THROUGH OPTICAL SYSTEM

λ (nm)	250	300	400	500	600	700
P (μW)	40	90	190	225	210	175

$$P = E_T A \Phi_{\text{illumination}} T_{\text{illumination}} \quad (2)$$

where A is the illuminated area of the pinhole, $\Phi_{\text{illumination}}$ is the collection efficiency for the illuminating optics and $T_{\text{illumination}}$ is the transmission of the illumination filter. For a system with illuminated area of $8 \cdot 10^{-9}$ m^2 (50-μm radius pinhole), collection efficiency of π steradians (25%), and average filter transmission of 50%, then the optical power delivered to the sample is given in Table II. That is, with a reasonable optical system, it appears possible to use a lamp to *deliver* microwatts of optical power (in a 10-nm spectral bandwidth) to a highly miniaturized fluroescence detector.

Use of a higher-power lamp is neither desirable nor useful in capillary electrophoresis. Xenon arc lamps tend to operate at constant temperature, independent of power; increased optical power is produced by increasing the size of the arc. After imaging the arc onto an appropriate size limiting aperture, the power delivered to the sample is independent of the lamp power, as long as the arc is larger than the limiting aperture. The increased power from a higher power lamp is lost on the limiting aperture.

EXPERIMENTAL

Illumination system

Fig. 1 shows a block diagram of the optical system. We use a 75-W xenon arc lamp (ILC Technology, Sunnyvale, CA, USA) for excitation. A Model 03 MHG 009 cold mirror (Melles Griot, Nepean, Canada) eliminates infrared radiation by reflection at 45° from the optical axis. This filter transmits visible light of wavelength shorter than 650 nm. An aspheric lens (Melles Griot Model 01 LAG 019) images the arc onto a 400 μm diameter pinhole placed at the focal point of the aspheric lens. In front of

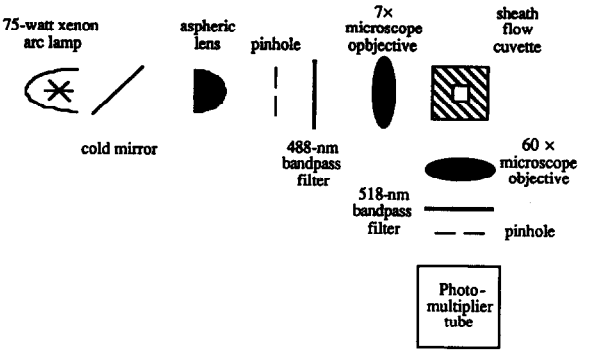

Fig. 1. Filter fluorometer. Light from a 75-W xenon arc lamp is filtered with a cold mirror, which reflects infrared radiation from the optical path. An aspheric lens is used to image the arc onto a 400-μm diameter pinhole. The transmitted radiation is spectrally filtered with a 10-nm bandpass interference filter centered at 488 nm. A 7× microscope objective images the pinhole into a sheath flow cuvette. Fluorescence is collected with a 60× microscope objective, filtered with a 518-nm bandpass filter, imaged onto an adjustable pinhole, and detected with a photomultiplier tube.

the pinhole, we use a 488-nm, 10-nm FWHM bandpass filter (Model 488 DF10; Omega Scientific, Burlington, VT, USA) to select the excitation wavelength. The pinhole is imaged onto the fluroescence detection chamber with a 7 × [N.A. (numerical aperture) 0.20] microscope objective (Melles Griot Model 04O AS 013), which produces a spot about 180 μm in diameter. This objective is 5.0 cm in front of the aspheric lens, mounted on a mirror holder that provides vertical adjustment and a Model 421-1S two-axis translation stage (Newport Instruments, Mississauga, Canada) to provide horizontal adjustment. An aluminum cover encloses the illumination system to minimize stray light. Additional baffles provide an effective blockage of all light not going through the microscope objective.

Post-column fluorescence detector

The quartz sheath flow cuvette is mounted onto a three-axis translation stage (constructed from three Newport Model 421-1S). The sheath flow chamber (NSG-Precision Cells, Farmingdale, NY, USA) has a square cross section of 0.04 mm^2 with 1-mm thick windows; the stainless-steel holder for the flow chamber is constructed locally. The illuminated volume is *ca.* $8 \cdot 10^{-3}$ mm^3. Adjustment of the height differ-

ence between the buffer reservoir and waste reservoir (3 cm approximately) allows control of the sheath flow. The fused-silica separation capillary is placed within the flow chamber; the excitation region is *ca.* 200 μm down stream from the capillary tip. The polyimide coating at the tip of the capillary inside the sheath flow cuvette has been previously eliminated using a gentle flame.

Collection optics and detection

Fluorescence is collected at right angles to the excitation source with a Model 60X-LWD 60× (N.A. 0.70) microscope objective (Universe Kogaku, Japan). To select fluorescence, we use a 518-nm, 25-nm FWHM bandpass filter (Omega Model 520 DF 25). The field of view is set by adjusting an iris located at the image plane of the objective. We align the components by observing visually the fluorescence from a 10^{-6} *M* fluorescein solution injected continuously at +20 000 V with an eyepiece (10×) instead of a detector. When alignment is optimized, the eyepiece is replaced by a water-cooled photomultiplier tube (Model R1477; Hamamatsu Photonics, NJ, USA) operated at −1200 V. We do a final alignment of the optical components by monitoring the photomultiplier tube output while injecting continuously 10^{-8} *M* fluorescein solution at +20 kV.

Capillary electrophoresis

The injection end of the capillary is less than 1 mm apart from the high voltage electrode (up to +29 000 V) inside a Plexiglas safety box. The other end of the capillary is inside the grounded sheath flow cuvette. The high-voltage power supply for electropherogram development and electrokinetic injection is controlled from a Macintosh IIsi computer with a multipurpose I/O board (National Instruments model MIO16XH).

Signal processing

The photomultiplier tube output is filtered (bandwidth 10 Hz) before it is processed by a Macintosh IIsi. The rate of data acquisition is 10 Hz. Data are displayed in real time using a Lab View 2.2 program and stored in binary files. The data are convoluted with a Gaussian filter characterized by a 0.5-s standard deviation.

Reagents

Stock $8.0 \cdot 10^{-3}$ *M* fluorescein (Molecular Probes, OR, USA) solution was prepared in 98% ethanol. Dilutions were prepared in pH 9.2, 5 m*M* borate buffer. $5.0 \cdot 10^{-3}$ *M* amino acid stock solutions were prepared in 0.2 *M* borate buffer. Fluorescein isothiocyanate (FITC; Sigma, MO, USA) derivatives were produced by mixing $1.55 \cdot 10^{-2}$ *M* FITC stock solution in 98% ethanol with amino acid stock solution in a 1:5 mol ratio and allowing to stand overnight. Dilutions and mixtures of different amino acids were prepared in pH 9.2, 5 m*M* borate + 10 m*M* sodium dodecyl sulfate buffer.

RESULTS AND DISCUSSION

We employ a sheath flow cuvette for post-column detection in our fluorescence detector. The excellent optical properties of the cuvette minimize light scatter, producing low background signals. In previous applications of the cuvette to fluorescence detection in capillary electrophoresis, only laser-based detection has been used [17–25]. Incoherent light sources have been used to excite fluorescence in flow cytometry, a technique which also used the sheath flow cuvette. Van Dilla *et al.* [34] reported the use of a mercury arc source to excite fluorescence in the sheath flow cuvette. Block Engineering used an incoherent light source to excite fluorescence in a flow cytometer based on a sheath flow cuvette [35]. In that instrument, a number of optical beams were used to illuminate the sample. Both a high pressure xenon arc lamp and a mercury arc lamp were used to generate fluorescence in an epilumination configuration.

Our fluorescence system was evaluated with both a 50-μm and 10-μm inner diameter capillary. Because of the large dimension of the sample stream produced by the 50-μm capillary, a rather large aperture was used to collect fluorescence. This large aperture also transmitted fluorescence from the polyimide coating of the capillary, producing unacceptably large background signals. By removing the capillary coat-

TABLE III

LIMITS OF DETECTION (LOD) FOR FLUORESCEIN

	10 μm I.D. capillary	50 μm I.D. capillary
Volume injected (l)	$6.8 \cdot 10^{-11}$	$1.8 \cdot 10^{-9}$
Fluorescein concentration (M)	$8.0 \cdot 10^{-10}$	$8.0 \cdot 10^{-11}$
LOD (M)	$2.0 \cdot 10^{-10}$	$1.5 \cdot 10^{-11}$
LOD (mol)	$1.4 \cdot 10^{-20}$	$2.8 \cdot 10^{-20}$

ing from the last few millimeters with a gentle flame, this background fluorescence was eliminated.

Detection limits were estimated by injecting known amounts of dilute fluorescein solutions. Table III presents the detection limits (3σ), estimated by the method of Knoll [36]. The mass detection limits are similar for the two capillaries; the 50-μm capillary produced superior concentration detection limits because of the use of a larger injection volume. These mass detection limits are roughly an order of magnitude inferior to those produced with an argon ion laser and a sheath flow cuvette [19]. The low excitation power produced by the filter fluorometer, along with the relatively large amount of stray light generated by the system, degrade the system performance compared with laser excitation. On the other hand, these fluorescence detection limits would have been the state-of-the-art in 1988. Improvements in stray light rejection and in illumination efficiency will certainly improve the performance of the filter fluorometer.

The detection limits produced by this filter fluorometer are more than two orders of magnitude superior to previous reports of lamp-based fluorescence detection. The improvement in performance results both from improved excitation efficiency and by use of the sheath flow cuvette to reduce scattered light from the capillary.

The linear dynamic range was estimated by injection of a series of fluorescein solutions. The response is linear ($r > 0.999$, $n = 5$) from the detection limit up to the saturation of the photomultiplier. The dynamic range extends from $8 \cdot 10^{-10}$ to $8 \cdot 10^{-7}$ M when the 10 μm I.D. capillary is used and from $2 \cdot 10^{-11}$ to $8 \cdot 10^{-7}$ M when the 50 μm I.D. capillary is used.

The rather large illumination volume produced by the filter fluorometer degraded the separation efficiency compared with that observed in laser-based detection. For $8 \cdot 10^{-10}$ M fluorescein, plate counts of 10^5 were observed for both the 10-μm and 50-μm inner diameter capillaries.

The post-column detector was applied to the separation of a nine FITC labeled acid mixture. Table IV lists the concentration and mass injected for the electropherogram in Fig. 3. The retention time for these derivatives is roughly three times greater than for fluorescein in Fig. 2, which presumably reflects differences in the ionization state of the molecules. The theoretical plate counts range from 150 000 to 200 000, and are typical for electrophoretic separation of these compounds.

Detection limits (3σ) for the glycine-fluorescein thiocarbamyl derivative are $2 \cdot 10^{-19}$ mol injected onto the capillary. These detection

TABLE IV

CONCENTRATION AND MASS INJECTED FOR FIG. 3

	Concentration (mol/l)	Mass injected (mol) × 10^{17}
Alanine	$1.5 \cdot 10^{-5}$	27
Aspartate	$9.6 \cdot 10^{-6}$	35
Lysine	$2.2 \cdot 10^{-6}$	3.1
Glycine	$2.0 \cdot 10^{-6}$	3.8
Isoleucine	$4.9 \cdot 10^{-6}$	8.6
Methionine	$1.6 \cdot 10^{-6}$	2.9
Phenylalanine	$9.0 \cdot 10^{-7}$	1.6
Threonine	$1.6 \cdot 10^{-6}$	3.0
Tryptophan	$4.4 \cdot 10^{-6}$	8.0

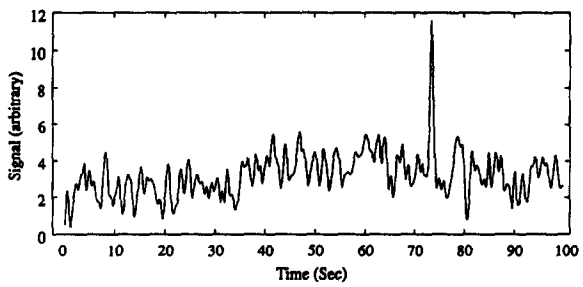

Fig. 2. Injection of $8 \cdot 10^{-10}$ *M* fluorescein. The separation was performed in a 37 cm × 10 μm I.D. capillary. Injection was for 5 s at 1 kV. Electrophoresis proceeded at 29 kV.

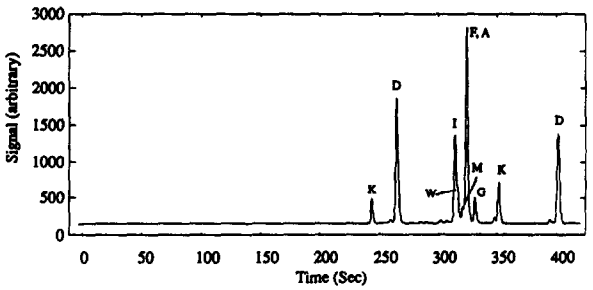

Fig. 3. Electropherogram of 9 fluorescein thiocarbamyl-amino acids. The separation was performed in a 37 cm × 10 μm I.D. capillary. Injection was for 2.5 s at 250 V. Electrophoresis proceeded at 20 kV in a pH 9.2, 5 m*M* borate and 10 m*M* sodium dodecyl sulfate buffer. The concentration and one-letter abbreviation of the derivatives are alanine (A) $1.5 \cdot 10^{-5}$ *M*, aspartate (D) $9.6 \cdot 10^{-6}$ *M*, lysine (K) $2.2 \cdot 10^{-6}$ *M*, glycine (G) $2.0 \cdot 10^{-6}$ *M*, isoleucine (I) $4.9 \cdot 10^{-6}$ *M*, methionine (M) $1.6 \cdot 10^{-6}$ *M*, phenylalanine (F) $9.0 \cdot 10^{-7}$ *M*, tryptophan (W) $4.4 \cdot 10^{-6}$ *M*.

limits are an order of magnitude poorer than those observed for fluorescein. The difference in performance is associated with the longer retention time for the derivative, a factor of two poorer quantum efficiency for FITC derivatives compared with native fluorescein [37], incomplete reaction between fluorescein and glycine, and degradation of the derivatizing reagent.

CONCLUSIONS

High-sensitivity fluorescence detection in capillary electrophoresis is possible without use of a laser. A low power arc lamp can deliver sufficient power to the small detection volume to produce detection limits in the mid-zeptomol range. By appropriate choice of excitation and emission filters, it should be possible to study a wide range of fluorescent materials with a single instrument.

The instrument performance results from several design choices. Excitation with a 75-W xenon arc lamp, with an integral parabolic reflector, efficiently couples light to the experiment. An aspheric lens efficiently illuminates the limiting aperture. A high-quality microscope objective images the aperture onto the cuvette. The sheath flow cuvette has excellent optical properties, resulting in background scatter that is lower than would be produced with on-column detection. A high quality microscope objective collects fluorescence from the sample. When combined with an aperture located at the image plane of the objective, light scatter is reduced dramatically. Further improvements in energy transfer from the lamp to the cuvette would result in gains in sensitivity, with ultimate performance near 1 zeptomol.

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